



## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

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<b>(54) Title:</b> ISOLATED PEPTIDES WHICH COMPLEX WITH HLA-Cw*16 MOLECULES, AND USES THEREOF  <b>(57) Abstract</b>  The invention involves the identification of peptides which complex with HLA-Cw*16 molecules, and which may then provoke lysis of the cells to which they bind, by cytolytic T cells. Diagnostic and therapeutic uses are described.		

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ISOLATED PEPTIDES WHICH COMPLEX WITH  
HLA-Cw\*16 MOLECULES, AND USES THEREOF

FIELD OF THE INVENTION

5 This invention relates to immunogenetics and to  
peptide chemistry. More particularly, it relates to  
peptides useful in various ways, including immunogens and  
as ligands for HLA-Cw\*16 molecules. More particularly, it  
relates to so-called "tumor rejection antigens", derived  
from the tumor rejection antigen precursor encoded by gene  
10 MAGE-6, and presented by molecules of MHC class HLA-Cw\*16.

BACKGROUND AND PRIOR ART

The study of the recognition or lack of recognition of  
cancer cells by a host organism has proceeded in many  
different directions. Understanding of the field presumes  
15 some understanding of both basic immunology and oncology.

Early research on mouse tumors revealed that these  
displayed molecules which led to rejection of tumor cells  
when transplanted into syngeneic animals. These  
molecules are "recognized" by T-cells in the recipient  
20 animal, and provoke a cytolytic T-cell response with lysis  
of the transplanted cells. This evidence was first  
obtained with tumors induced in vitro by chemical  
carcinogens, such as methylcholanthrene. The antigens  
expressed by the tumors and which elicited the T-cell  
25 response were found to be different for each tumor. See  
Prehn, et al., J. Natl. Canc. Inst. 18: 769-778 (1957);  
Klein et al., Cancer Res. 20: 1561-1572 (1960); Gross,  
Cancer Res. 3: 326-333 (1943), Basombrio, Cancer Res. 30:  
2458-2462 (1970) for general teachings on inducing tumors  
30 with chemical carcinogens and differences in cell surface  
antigens. This class of antigens has come to be known as  
"tumor specific transplantation antigens" or "TSTAs".  
Following the observation of the presentation of such  
antigens when induced by chemical carcinogens, similar  
35 results were obtained when tumors were induced in vitro via

ultraviolet radiation. See Kripke, J. Natl. Canc. Inst. 53: 333-1336 (1974).

5 While T-cell mediated immune responses were observed for the types of tumor described supra, spontaneous tumors were thought to be generally non-immunogenic. These were therefore believed not to present antigens which provoked a response to the tumor in the tumor carrying subject. See Hewitt, et al., Brit. J. Cancer 33: 241-259 (1976).

10 The family of tum antigen presenting cell lines are immunogenic variants obtained by mutagenesis of mouse tumor cells or cell lines, as described by Boon et al., J. Exp. Med. 152: 1184-1193 (1980), the disclosure of which is incorporated by reference. To elaborate, tum antigens are obtained by mutating tumor cells which do not generate an  
15 immune response in syngeneic mice and will form tumors (i.e., "tum<sup>+</sup>" cells). When these tum<sup>+</sup> cells are mutagenized, they are rejected by syngeneic mice, and fail to form tumors (thus "tum"). See Boon et al., Proc. Natl. Acad. Sci. USA 74: 272 (1977), the disclosure of which is  
20 incorporated by reference. Many tumor types have been shown to exhibit this phenomenon. See, e.g., Frost et al., Cancer Res. 43: 125 (1983).

It appears that tum variants fail to form progressive tumors because they initiate an immune rejection process.  
25 The evidence in favor of this hypothesis includes the ability of "tum" variants of tumors, i.e., those which do not normally form tumors, to do so in mice with immune systems suppressed by sublethal irradiation, Van Pel et al., Proc. Natl. Acad. Sci. USA 76: 5282-5285 (1979); and  
30 the observation that intraperitoneally injected tum cells of mastocytoma P815 multiply exponentially for 12-15 days, and then are eliminated in only a few days in the midst of an influx of lymphocytes and macrophages (Uyttenhove et al., J. Exp. Med. 152: 1175-1183 (1980)). Further evidence  
35 includes the observation that mice acquire an immune memory which permits them to resist subsequent challenge to the

same tumor variant, even when immunosuppressive amounts of radiation are administered with the following challenge of cells (Boon et al., Proc. Natl. Acad. Sci. USA 74: 272-275 (1977); Van Pel et al., supra; Uyttenhove et al., supra).

5 Later research found that when spontaneous tumors were subjected to mutagenesis, immunogenic variants were produced which did generate a response. Indeed, these variants were able to elicit an immune protective response against the original tumor. See Van Pel et al., J. Exp.  
10 Med. 157: 1992-2001 (1983). Thus, it has been shown that it is possible to elicit presentation of a so-called "tumor rejection antigen" in a tumor which is a target for a syngeneic rejection response. Similar results have been obtained when foreign genes have been transfected into  
15 spontaneous tumors. See Fearon et al., Cancer Res. 48: 2975-1980 (1988) in this regard.

A class of antigens has been recognized which are presented on the surface of tumor cells and are recognized by cytolytic T cells, leading to lysis. This class of  
20 antigens will be referred to as "tumor rejection antigens" or "TRAs" hereafter. TRAs may or may not elicit antibody responses. The extent to which these antigens have been studied, has been via cytolytic T cell characterization studies, in vitro i.e., the study of the identification of  
25 the antigen by a particular cytolytic T cell ("CTL" hereafter) subset. The subset proliferates upon recognition of the presented tumor rejection antigen, and the cells presenting the tumor rejection antigens are lysed. Characterization studies have identified CTL clones  
30 which specifically lyse cells expressing the tumor rejection antigens. Examples of this work may be found in Levy et al., Adv. Cancer Res. 24: 1-59 (1977); Boon et al., J. Exp. Med. 152: 1184-1193 (1980); Brunner et al., J. Immunol. 124: 1627-1634 (1980); Maryanski et al., Eur. J. Immunol. 124: 1627-1634 (1980); Maryanski et al., Eur. J. Immunol. 12: 406-412 (1982); Palladino et al., Canc. Res. 47: 5074-5079 (1987). This type of analysis is  
35

required for other types of antigens recognized by CTLs, including minor histocompatibility antigens, the male specific H-Y antigens, and the class of antigens referred to as "tum-" antigens, and discussed herein.

5 A tumor exemplary of the subject matter described supra is known as P815. See DePlaen et al., Proc. Natl. Acad. Sci. USA 85: 2274-2278 (1988); Szikora et al., EMBO J 9: 1041-1050 (1990), and Sibille et al., J. Exp. Med. 172: 35-45 (1990), the disclosures of which are  
10 incorporated by reference. The P815 tumor is a mastocytoma, induced in a DBA/2 mouse with methylcholanthrene and cultured as both an in vitro tumor and a cell line. The P815 line has generated many tum variants following mutagenesis, including variants referred  
15 to as P91A (DePlaen, supra), 35B (Szikora, supra), and P198 (Sibille, supra). In contrast to tumor rejection antigens - and this is a key distinction - the tum antigens are only present after the tumor cells are mutagenized. Tumor rejection antigens are present on cells of a given tumor  
20 without mutagenesis. Hence, with reference to the literature, a cell line can be tum<sup>+</sup>, such as the line referred to as "P1", and can be provoked to produce tum variants. Since the tum phenotype differs from that of the parent cell line, one expects a difference in the DNA of  
25 tum cell lines as compared to their tum<sup>+</sup> parental lines, and this difference can be exploited to locate the gene of interest in tum cells. As a result, it was found that genes of tum variants such as P91A, 35B and P198 differ from their normal alleles by point mutations in the coding  
30 regions of the gene. See Szikora and Sibille, supra, and Lurquin et al., Cell 58: 293-303 (1989). This has proved not to be the case with the TRAs of this invention. These papers also demonstrated that peptides derived from the tum antigen are presented by the L<sup>d</sup> molecule for recognition by  
35 CTLs. P91A is presented by L<sup>d</sup>, P35 by D<sup>d</sup> and P198 by K<sup>d</sup>.

PCT application PCT/US92/04354, filed on May 22, 1992 assigned to the same assignee as the subject application, teaches a family of human tumor rejection antigen precursor coding genes, referred to as the MAGE family. Several of these genes are also discussed in van der Bruggen et al., Science 254: 1643 (1991). It is now clear that the various genes of the MAGE family are expressed in tumor cells, and can serve as markers for the diagnosis of such tumors, as well as for other purposes discussed therein. See also Traversari et al., Immunogenetics 35: 145 (1992); van der Bruggen et al., Science 254: 1643 (1991) and De Plaen, et al., Immunogenetics 40: 360 (1994). The mechanism by which a protein is processed and presented on a cell surface has now been fairly well documented. A cursory review of the development of the field may be found in Barinaga, "Getting Some 'Backbone': How MHC Binds Peptides", Science 257: 880 (1992); also, see Fremont et al., Science 257: 919 (1992); Matsumura et al., Science 257: 927 (1992); Latron et al., Science 257: 964 (1992). These papers generally point to a requirement that the peptide which binds to an MHC/HLA molecule be nine amino acids long (a "nonapeptide"), and to the importance of the first and ninth residues of the nonapeptide.

Studies on the MAGE family of genes have now revealed that a particular nonapeptide is in fact presented on the surface of some tumor cells, and that the presentation of the nonapeptide requires that the presenting molecule be HLA-A1. Complexes of the MAGE-1 tumor rejection antigen (the "TRA" or nonapeptide) leads to lysis of the cell presenting it by cytolytic T cells ("CTLs").

Attention is drawn, e.g., to applications Serial No. 08/217,188 to Melief et al, Serial No. 08/217,187 to Traversari et al., and Serial No. 08/217,186 to Townsend et al., all of which present work on other, MAGE-derived peptides.

Research presented in, e.g., U.S. patent application Serial No. 07/938,334 filed August 31, 1992, and in U.S.

patent application Serial No. 073,103, filed June 7, 1993, found that when comparing homologous regions of various MAGE genes to the region of the MAGE-1 gene coding for the relevant nonapeptide, there is a great deal of homology. Indeed, these observations lead to one of the aspects of the invention disclosed and claimed therein, which is a family of nonapeptides all of which have the same N-terminal and C-terminal amino acids. These nonapeptides were described as being useful for various purposes which includes their use as immunogens, either alone or coupled to carrier peptides. Nonapeptides are of sufficient size to constitute an antigenic epitope, and the antibodies generated thereto were described as being useful for identifying the nonapeptide, either as it exists alone, or as part of a larger polypeptide.

The human major histocompatibility complex (MHC) system is an involved one. One feature of the system is the human leukocyte antigen, or "HLA". Human cells can be "typed", based upon their HLA profile. Not all cells present all types of HLA molecules. The diversity of this system may be seen by reference to, e.g., Zemmour et al., Immunogenetics 37: 239-250 (1993). This reference shows that, as of 1992, there were literally dozens of different HLA alleles which were known to the art. Cianetti et al., Immunogenetics 29: 80-91 (1989), the disclosure of which is incorporated by reference, discloses, inter alia, an MHC allele referred to therein as HLA-C-clone 10. This allele was later renamed, as per Bodmer et al., Tissue Antigen 44: 1-18 (1994), incorporated by reference. The allele is now known as HLA-Cw\*1601. A related allele, i.e., HLA-Cw\*1602 is also known. See Bodmer et al, supra; Vilches et al., Human Immunol. 41: 167-170 (1994), also incorporated by reference. These two alleles, when discussed collectively, will be referred to as "HLA-Cw\*16". Van der Bruggen et al, Eur. J. Immunol. 24: 2134-2140 (1994), incorporated by reference, teach that cytolytic T cells ("CTLs") recognize a complex of a peptide and an HLA-Cw\*1601 molecule. These



data are also disclosed in copending U.S. patent application Serial Number 08/79,110, filed June 17, 1993, now allowed and incorporated by reference, and in U.S. patent application Serial Number 08/196,630, filed on  
5 February 15, 1994, and incorporated by reference. Both applications are assigned to the assignee of the subject application. These two applications disclose that the tumor rejection antigen precursor referred to as "BAGE" is processed to peptides presented by the HLA-Cw\*1601 molecule.  
10 Specifically, the applications disclose a preferred nonapeptide:

Ala Ala Arg Ala Val Phe Leu Ala Leu  
(SEQ ID NO: 1), which complexes with HLA-Cw\*1601, thereby stimulating the cell line CTL 82/82.

15 U.S. Patent No. 5,342,774, incorporated by reference, discloses a family of related nucleic acid molecules which encode tumor rejection antigen precursors referred to as the MAGE tumor rejection antigen precursor. These "TRAPS" are numbered as MAGE-1, MAGE-2, etc. Generally, they are  
20 expressed predominantly on tumor cells, normal testes cells being the major exception, and they are processed to peptides presented by various HLA molecules, such as HLA-A1, HLA-A2, and so forth. See, e.g., U.S. Patent No. 5,405,940, incorporated by reference.

25 Not all tumor cells express all MAGE TRAPs. Hence, while it is desirable for the field to have information available on specific complexes of peptide and HLA molecules which do stimulate T cells, this is not always easy to secure. One requires a cell which expresses both  
30 the required MHC molecule and the required TRAP molecule, and the ability to process the large TRAP molecule to the smaller, tumor rejection antigen or "TRA". Even when a particular peptide binds with an MHC molecule to form a complex, this does not per se guarantee that the complex  
35 will stimulate CTL proliferation. In other words while peptide MHC binding is sufficient to identify MHC phenotype

on a cell, this binding is necessary but not sufficient to provoke CTL proliferation.

5 The inventors have found, however, that the TRAP encoded by the MAGE-6 gene is processed to TRAs which bind to MHC Molecules of type HLA-Cw\*16, and also provoke proliferation of CTLs thereby. Hence, the invention is directed, inter alia, to these peptides and their various uses, as will be explained in the disclosure which follows.

#### BRIEF DESCRIPTION OF THE FIGURES

10 Figure 1 present data showing that cytolytic T cell line CTL 82/21 lyses lines MZ2-MEL.43 and MZ2-MEL.3.0, but not line MZ2-MEL.3.1, nor does it lyse natural killer cell line K562.

15 Figure 2 sets forth results of assays to determine if various cell lines, transfected, cotransfected, and nontransfected, stimulate TNF release by CTL 82/21.

Figure 3 shows the result of lysis studies, using peptides derived from SEQ ID NO: 2.

#### DETAILED DESCRIPTION OF PREFERRED EMBODIMENTS

##### 20 Example 1

Previous work by, e.g., Van den Eynde et al., Int. J. Cancer 44: 634-640 (1989), incorporated by reference, has shown that human melanoma cells present multiple antigens, recognized by different cytolytic T cell lines ("CTLs" hereafter). Four such antigens are referred to by Van den Eynde et al., supra, as MZ2-B, D, E and F. One such CTL is referred to as MZ2-CTL 82/21, and it was used in the experiments which follow.

30 One well known melanoma cell line is MZ2-MEL, which was derived from an abdominal metastasis. This cell line was then subjected to limiting dilution to yield subline MZ2-MEL.3.0. The subline, in turn, was cultured for over 150 generations to yield subline MZ2-MEL.3.1. An

additional subline, MZ2-MEL.43, was prepared by subjecting MZ2-MEL.3.0 to a mutagen, and then limiting dilution. See Herin et al, Int. J. Cancer 39: 390-396 (1987); Van den Eynde, supra. The three cell lines MZ2-MEL.3.0, MZ2-MEL.3.1, and MZ2-MEL.43 were then used, together with killer cell line K562, in a <sup>51</sup>Cr release assay, following Traversari, et al., Immunogenetics 35: 145-152 (1992), and Boon et al., J. Exp. Med. 152: 1184-1193 (1980), using CTL 82/21, supra.

The results, shown in figure 1, suggest, inter alia, that MZ2-MEL.3.1 has lost the antigen targeted by CTL 82/21, but that this antigen is found on both MZ2-MEL.3.0 and MZ2-MEL.43. Analysis, in accordance with van der Bruggen et al., Eur. J. Immunol. 24: 2134-2140 (1994), revealed that this cell line had lost the genes encoding HLA-A29, HLA-B44, and HLA-Cw\*1601, which differentiated it from both MZ2-MEL.3.0 and MZ2-MEL.3.1. This led to the conclusion that one of these three listed MHC molecules was responsible for the presentation of a peptide which, in combination with the MHC Molecule, led to recognition by CTL 82/21.

#### Example 2

A cDNA sequence encoding HLA-Cw\*1601 had been isolated previously, as can be seen in van der Bruggen et al., Eur. J. Immunol. 24: 2134-2140 (1994), incorporated by reference. A cell line which was known to have lost expression of HLA-Cw\*1601 was known and available, i.e., MZ2-MEL.2.2.5. This cell line was also known to have lost expression of MAGE-1 (MZ2-MEL.2.2.5 is a subline of MZ2-MEL.2.2, which does express MAGE-1). MZ2 MEL-2.2.5 does express MAGE-2, 3, 6 and 12. As a result, it was a suitable cell line for determining whether or not HLA-Cw\*1601, in combination with a peptide, would provoke lysis by cytolytic T cell CTL 82/21.

HLA-Cw\*1601 cDNA was inserted into plasmid pcDSR $\alpha$  in accordance with van der Bruggen, et al., Eur. J. Immunol. 24: 2134-2140 (1994). MAGE-1 cDNA was inserted into plasmid pcDNA1 via an EcoRI site. Two different plasmids were thus established. The two plasmids were cotransfected into MZ2-MEL2.2.5, together with a plasmid which imparts resistance to G418 (i.e., pSVtkneo8), in accordance with the calcium phosphate method of van der Bruggen et al., Science 254: 1634 (1991).

Transfectants were first selected for resistance to G418, and then tested for expression of MAGE-1 and HLA-Cw\*1601 using CTL 81/21, by determining whether or not they could stimulate TNF production in accordance with Traversari et al., Immunogenetics 35: 145-152 (1992). In brief, 1500 CTLs were added to 100  $\mu$ l Iscove's medium supplemented with 10% human serum and 25 U/ml recombinant human IL-2. This mixture was combined with transfected MZ-MEL.2.2.5 cells, cultured for 24 hours, and then the supernatant was collected. The TNF content was determined by testing the cytotoxicity on WEHI-164 clone 13 cells, in accordance with Espevik et al., J. Immunol. Meth. 95: 99-105 (1986), in an MTT colorimetric assay as described by Hansen et al., J. Immunol. Meth. 119: 203-21- (1989); Traversari et al., Immunogenetics 35: 145-152 (1992).

The first three bars of figure 2 show these results, using cell lines MZ2-MEL.43 and MZ2-MEL.3.1 as controls. The transfection of MZ2-2.2.5 clearly resulted in stimulation of CTL 82/21. What was not clear from this work, however, was whether or not MAGE-1 was necessary for the stimulation.

### Example 3

To clarify the issue raised following the experiments in example 2, COS-7 cells were transfected by the DEAE-Dextran chloroquine method of Brichard et al., J. Exp. Med. 178: 489-495 (1993); Coulie, et al., J. Exp. Med. 180: 35-42 (1994); Seed et al., Proc. Natl. Acad. Sci. USA 84:

3365-3369 (1987), and tested in the same manner the MZ2-MEL.2.2.5 cells were tested. In addition to MAGE-1, other TRAP encoding cDNAs were used, including MAGE-2, 3, 4 and 12, BAGE, Melan-A, tyrosinase, and GAGE-1, 2, 3, 4, 5 and 6. As figure 2 shows, none of these transfectants led to stimulation of CTL 82/21. As was noted, *supra*, MZ2-MEL.2.2.5 expresses MAGE-2, 3, 6 and 12 naturally, and was transfected to express MAGE-1. One of the differences between the two sets of experiments is the presence of MAGE-6 in the MZ2-MEL.2.2.5 cell line.

#### Example 4

As a follow up to the experiments of example 3, a cDNA library was prepared from MZ2-MEL.43, and inserted into pCDSR $\alpha$ , using standard methods. The library was transfected into COS cells, together with a plasmid containing HLA-Cw\*1601 in pCDSR $\alpha$ , using the methodologies of example 2, *supra*, and were then tested for TNF release, also as described, *supra*. Two positive clones were obtained. The plasmid DNA for the positive clones were electroporated into bacteria (E. coli DH5 $\alpha$ ), using well known methods.

Following the identification of positive colonies, the plasmid DNA was extracted, and about 1/3 was transfected into COS-7 cells together with the HLA-Cw\*1601 construct. Clone GEP 3/317 2/B7 conferred recognition by CTL 82/21. Upon sequencing, the clone was identified as MAGE-6, as described by DePlaen et al., Immunogenetics 40: 360-369 (1994), incorporated by reference. SEQ ID NO: 2 sets this sequence forth.

#### Example 5

As MAGE-6 was identified as the TRAP which was processed to the presented peptide, studies were carried out to determine the particular peptide of relevance.

To do this, PCR primers:

GACCAGAGTC ATCATGCCTC T (SEQ ID NO: 3)

and

TCCTCCACTG ATCTTTACC (SEQ ID NO: 4)

were used. These were sense and antisense primers, respectively, corresponding to nucleotides 147-167 and 1029-1047 of cDNA for MAGE-6 of SEQ ID NO: 2.

5 PCR amplification was carried out on clone GEP 3/317 2/B7. After four minutes at 94°C, PCR was carried out for 20 cycles (1 cycle: 1 minute at 94°C, 2 minutes at 60°C, and 2 minutes at 72°C), followed by 15 minutes at 72°C. 10 PCR product was then inserted into plasmid pCR3, using standard methods, and transfected into COS-7. The resulting transfectant did not code for the antigen.

When the truncated form was compared to the "base clone", i.e., GEP 3/317 2B/7, via sequence analysis, it was 15 noted that the PCR product consisted of nucleotides 147 to 1047 of the ORF, while the base clone consisted of nucleotides 160-1104, indicating that the antigenic peptide necessarily had a C-terminus in nucleotides 1047-1104.

Based upon this hypothesis, a series of peptides were 20 constructed, and lyophilized prior to testing. The lyophilized peptides were dissolved in 1 volume of DMSO, then 9 volumes of H<sub>2</sub>O, plus 10 mM of acetic acid. The thus diluted peptides were stored at -20°C.

The ability of the peptides to stimulate lysis by CTLs 25 was tested via the <sup>51</sup>Cr release method of Boon et al., J. Exp. Med. 152: 1184-1193 (1980). Specifically, an HLA-Cw\*1601 positive lymphoblastoid cell line, i.e., LB678-EBV was <sup>51</sup>Cr labelled for one hour at 37°C, and washed extensively. Then 1000 of these target cells were 30 incubated in 96 well microplates, in the presence of various concentrations of peptide, for 15 minutes, at room temperature. CTLs were then added, and <sup>51</sup>Cr release was measured after four hours, at 37°C.

Lysis first occurred with the peptide:

35 Lys Ile Ser Gly Gly Pro Arg Ile Ser Tyr Pro Leu

(SEQ ID NO: 5), which corresponds to amino acids 126-137 of MAGE-6. Shorter peptides were also tested, and it was found that the nonamer consisting of amino acids 2-10 of SEQ ID NO: 5 achieved half maximal lysis at a peptide concentration of 30 nM. This is seen in figure 3.

The nonapeptide corresponds to a sequence found in MAGE-3, with one exception, i.e., the Arg of the nonapeptide is His in MAGE-3. A nonapeptide corresponding to the MAGE-3 sequence was synthesized, and tested, but was found not to stimulate lysis. Similarly, when peptides were tested which consisted of amino acids 1-9 of SEQ ID NO: 5, and amino acids 3-10 of SEQ ID NO: 5, it was found that neither one stimulated lysis.

Thus, peptides which bind to HLA-Cw\*1601 which bind to HLA-Cw\*1601 may be defined as peptides where Ile is N-terminal to Tyr, and these two residues are separated by seven amino acids. The fifth of these separating amino acids must be Arg, and Ile does not have to be the N-terminus of the peptide, nor does Tyr have to be the C-terminus because, as noted supra, the peptide of SEQ ID NO: 4 does bind to HLA-Cw\*1601 and provokes lysis by cytolytic T cells.

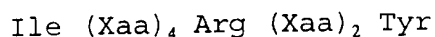
Hence, one aspect of the invention are isolated peptides of formula:

$$\text{NH}_2\text{-Xaa Ile (Xaa)}_4 \text{Arg (Xaa)}_2 \text{Tyr Xaa-COOH}$$

(SEQ ID NO: 6), wherein N terminal Xaa and C-terminal Xaa are anywhere from 0 to 3 amino acids in total, and can be any amino acid. Especially preferred are molecules such as SEQ ID NO: 5, wherein N-terminal Xaa is one amino acid, and C-terminal Xaa is two amino acids. Also preferred are molecules where  $\text{NH}_2\text{-Xaa}$  and  $\text{Xaa-COOH}$  are not present, such as the peptide defined by amino acids 2-10 of SEQ ID NO: 5. These peptides are peptides which bind to MHC molecules of the HLA-Cw\*16 type, e.g., HLA-Cw\*1601 and HLA-Cw\*1602, and preferably provoke lysis by CTLs specific therefor. Thus, one can identify HLA-Cw\*16 presenting cells via use of these

peptides, including peptides labelled with a detectable signal. The peptides can also be used to provoke lysis of HLA-Cw\*1601 presenting cells, if the peptides are present in amounts sufficient to complex HLA-Cw\*16 molecules on cellular surfaces. This can be done via, e.g., contacting the cells with the peptides, or transforming or transfecting host cells with one of both of nucleic acid molecules encoding HLA-Cw\*16, and MAGE-6. Either or both of these may be gDNA or cDNA.

Some work has been done on binding motifs for HLA-C molecules, although it is by no means as developed as that which has been carried out for HLA-A molecules. See, e.g., Falk et al., Proc. Natl. Acad. Sci. USA 90: 12005-12009 (1993), and Ramensee et al., Immunogenetics 41: 178-228 (1995), both of which are incorporated by reference. These references, Falk in particular, suggest that in the case of nonamers which bind to HLA-C molecules, positions 3, 5 and 6 are important. Hence, with reference to SEQ ID NO: 6, especially preferred are molecules where NH<sub>2</sub>-Xaa and Xaa-COOH are absent, wherein the following formula results:



(SEQ ID NO: 7),

wherein within (Xaa)<sub>4</sub>, the second amino acid is Gly, and/or the fourth amino acid is Pro. Especially preferred are nonapeptides where the second Xaa in (Xaa)<sub>4</sub> is Gly and the fourth amino acid in (Xaa)<sub>4</sub> is Pro. All other residues may be any other amino acid.

In connection with this aspect of the invention, it was noted that nucleotides 1047 through 1104 of MAGE-6 cDNA, as set forth in SEQ ID NO: 2, encode at least the C-terminus of the pertinent tumor rejection antigen. The location of the antigenic peptide of SEQ ID NO: 5 was determined to start at nucleotide 1033 of SEQ ID NO: 2. Hence, a further aspect of this invention is the isolated nucleic acid molecule consisting of nucleotides 1033 through 1104 of SEQ ID NO: 2. This sequence is useful



alone, e.g., as a probe for determining expression of MAGE-6, or it can be used in an expression vector, operably linked to a promoter, to express the relevant tumor rejection antigen. Cells which have been transformed or  
5 transfected with this nucleic acid molecule, alone or in combination with nucleic acid molecules encoding an HLA-Cw\*16 encoding nucleic acid molecule are also a feature of this invention.

The identification of the TRA and the identification  
10 of its complexing MHC molecule makes it possible to develop a therapeutic method for treating subjects with disorders characterized by expression of HLA-Cw16 molecules, such as SEQ ID NO: 5 when presented as the TRA, or as complexes of TRA and HLA, such as HLA-Cw\*16, may be combined with  
15 materials such as adjuvants to produce vaccines useful in treating disorders characterized by expression of the TRAP molecule. In addition, vaccines can be prepared from cells which present the TRA/HLA complexes on their surface, such as non-proliferative cancer cells, non-proliferative  
20 transfectants, etcetera. In all cases where cells are used as a vaccine, these can be cells transfected with coding sequences for one or both of the components necessary to prove a CTL response, or be cells which express both molecules without transfection. Further, the MAGE-6  
25 molecules, its associated TRAs, such as those disclosed herein as well as complexes of TRA and HLA, may be used to produce antibodies, using standard techniques well known to the art.

When "disorder" is used herein, it refers to any  
30 pathological condition where the tumor rejection antigen precursor is expressed. An example of such a disorder is cancer, melanoma in particular.

Therapeutic approaches based upon the disclosure are premised on a response by a subject's immune system,  
35 leading to lysis of TRA presenting cells, such as HLA-Cw\*16 presenting cells. One such approach is the administration of CTLs specific to the complex to a subject with abnormal

cells of the phenotype at issue. it is within the skill of the artisan to develop such CTLs in vitro. Specifically, a sample of cells, such as blood cells, are contacted to a cell presenting the complex and capable of provoking a specific CTL to proliferate. The target cell can be a transfectant, such as a COS cell of the type described supra. These transfectants present the desired complex on their surface and, when combined with a CTL of interest, stimulate its proliferation. COS cells, such as those used herein are widely available, as are other suitable host cells.

To detail the therapeutic methodology, referred to as adoptive transfer (Greenberg, J. Immunol. 136(5): 1917 (1986); Reddel et al., Science 257: 238 (7-10-92); Lynch et al., Eur. J. Immunol. 21: 1403-1410 (1991); Kast et al., Cell 59: 603-614 (11-17-89)), cells presenting the desired complex are combined with CTLs leading to proliferation of the CTLs specific thereto. The proliferated CTLs are then administered to a subject with a cellular abnormality which is characterized by certain of the abnormal cells presenting the particular complex. The CTLs then lyse the abnormal cells, thereby achieving the desired therapeutic goal.

The foregoing therapy assumes that at least some of the subject's abnormal cells present the relevant HLA/TRA complex. This can be determined very easily, as the art is very familiar with methods for identifying cells which present a particular HLA molecule, as well as how to identify cells expressing DNA of the pertinent sequences, in this case the MAGE-6 sequence. Once cells presenting the relevant complex are identified via the foregoing screening methodology, they can be combined with a sample from a patient, where the sample contains CTLs. If the complex presenting cells are lysed by the mixed CTL sample, then it can be assumed that a BAGE derived, tumor rejection antigen is being presented, and the subject is an

appropriate candidate for the therapeutic approaches set forth supra.

Adoptive transfer is not the only form of therapy that is available in accordance with the invention. CTLs can also be provoked in vivo, using a number of approaches. One approach, i.e., the use of non-proliferative cells expressing the complex, has been elaborated upon supra. The cells used in this approach may be those that normally express the complex, such as irradiated melanoma cells or cells transfected with one or both of the genes necessary for presentation of the complex. Chen et al., Proc. Natl. Acad. Sci. USA 88: 110-114 (January, 1991) exemplifies this approach, showing the use of transfected cells expressing HPVE7 peptides in a therapeutic regime. Various cell types may be used. Similarly, vectors carrying one or both of the genes of interest may be used. Viral or bacterial vectors are especially preferred. In these systems, the gene of interest is carried by, e.g., a Vaccinia virus or the bacteria BCG, and the materials de facto "infect" host cells. The cells which result present the complex of interest, and are recognized by autologous CTLs, which then proliferate. A similar effect can be achieved by combining the tumor rejection antigen or the precursor itself with an adjuvant to facilitate incorporation into HLA-Cw\*16 presenting cells which present the HLA molecule of interest. The TRAP is processed to yield the peptide partner of the HLA molecule while the TRA is presented without the need for further processing.

Other aspects of the invention will be clear to the skilled artisan and need not be repeated here.

The terms and expressions which have been employed are used as terms of description and not of limitation, and there is no intention in the use of such terms and expressions of excluding any equivalents of the features shown and described or portions thereof, it being recognized that various modifications are possible within the scope of the invention.

## (1) GENERAL INFORMATION:

- (i) APPLICANTS: van der Bruggen, Pierre; DePlaen Etienne;  
Boon-Falleur, Thierry
- (ii) TITLE OF INVENTION: Isolated Peptides Which Complex With  
HLA-Cw\*16 Molecules, and Uses Thereof
- (iii) NUMBER OF SEQUENCES: 9
- (iv) CORRESPONDENCE ADDRESS:
  - (A) ADDRESSEE: Felfe & Lynch
  - (B) STREET: 805 Third Avenue
  - (C) CITY: New York City
  - (D) STATE: New York
  - (F) ZIP: 10022
- (v) COMPUTER READABLE FORM:
  - (A) MEDIUM TYPE: Diskette, 3.5 inch, 360 kb storage
  - (B) COMPUTER: IBM
  - (C) OPERATING SYSTEM: PC-DOS
  - (D) SOFTWARE: Wordperfect
- (vi) CURRENT APPLICATION DATA:
  - (A) APPLICATION NUMBER:
  - (B) FILING DATE:
  - (C) CLASSIFICATION:
- (vii) PRIOR APPLICATION DATA:
  - (A) APPLICATION NUMBER: 08/713,354
  - (B) FILING DATE: 13-September-1996
  - (C) CLASSIFICATION: 435
- (viii) ATTORNEY/AGENT INFORMATION:
  - (A) NAME: Hanson, Norman D.
  - (B) REGISTRATION NUMBER: 30,946
  - (C) REFERENCE/DOCKET NUMBER: LUD 5460-PCT
- (ix) TELECOMMUNICATION INFORMATION:
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  - (B) TELEFAX: (212) 838-3884

19

- (2) INFORMATION FOR SEQUENCE ID NO: 1:  
 (i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 9 amino acid residues  
 (B) TYPE: amino acid  
 (D) TOPOLOGY: linear  
 (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

Ala Ala Arg Ala Val Phe Leu Ala Leu  
 5

- (2) INFORMATION FOR SEQUENCE ID NO: 2:  
 (i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 1375 nucleotides  
 (B) TYPE: nucleic acid  
 (C) STRANDEDNESS: single  
 (D) TOPOLOGY: linear  
 (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

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GCCGGCCCCAG GCTCGGTGAG GAGGCAAGGT TCTGAGGGGA CAGGCTGACC TGGAGGACCA 60
GAGGCCCCCG GAGGAGCACT GAAGGAGAAG ATCTGCCAGT GGGTCTCCAT TGCCCCAGCTC 120
CTGCCCCACAC TCCCGCCTGT TGCCCTGACC AGAGTCATCA TGCCTCTTGA GCAGAGGAGT 180
CAGCACTGCA AGCCTGAAGA AGGCCTTGAG GCCCGAGGAG AGGCCCTGGG CCTGGTGGGT 240
GCGCAGGCTC CTGCTACTGA GGAGCAGGAG GCTGCCTCCT CCTCTTCTAC TCTAGTTGAA 300
GTCACCCTGG GGGAGGTGCC TGCTGCCGAG TCACCAGATC CTCCCCAGAG TCCTCAGGGA 360
GCCTCCAGCC TCCCCACTAC CATGAACCTAC CCTCTCTGGA GCCAATCCTA TGAGGACTCC 420
AGCAACCAAG AAGAGGAGGG GCCAAGCACC TTCCCTGACC TGGAGTCTGA GTTCCAAGCA 480
GCACTCAGTA GGAAGGTGGC CAAGTTGGTT CATTTTCTGC TCCTCAAGTA TCGAGCCAGG 540
GAGCCGGTCA CAAAGGCAGA AATGCTGGGG AGTGTCGTCG GAAATTGGCA GTACTTCTTT 600
CCTGTGATCT TCAGCAAAGC TTCCGATTCC TTGCAGCTGG TCTTTGGCAT CGAGCTGATG 660
GAAGTGGACC CCATCGGCCA CGTGATACAT TTTGCCACCT GCCTGGGCCT CTCCTACGAT 720
GGCCTGCTGG GTGACAATCA GATCATGCCC AAGACAGGCT TCCTGATAAT CATCTGGCC 780
ATAATCGCAA AAGAGGGCGA CTGTGCCCCCT GAGGAGAAAA TCTGGGAGGA GCTGAGTGTG 840
TTAGAGGTGT TTGAGGGGAG GGAAGACAGT ATCTTCGGGG ATCCCAAGAA GCTGCTCACC 900
CAATATTTTCG TGCAGGAAAA CTACCTGGAG TACCGGCAGG TCCCCGGCAG TGATCCTGCA 960
TGCTATGAGT TCCTGTGGGG TCCAAGGCC CTCATTGAAA CCAGCTATGT GAAAGTCTTG 1020
CACCATATGG TAAAGATCAG TGGAGGACCT CGCATTTCCT ACCCACTCCT GCATGAGTGG 1080
GCTTTGAGAG AGGGGGAAGA GTGAGTCTGA GCACGAGTTG CAGCCAGGGC CAGTGGGAGG 1140
GGGTTTGGGC CAGTGCACCT TCCGGGGCCC CATCCCTTAG TTTCCACTGC CTCCTGTGAC 1200
GTGAGGCCCCA TTCTTCACTC TTTGAAGCGA GCAGTCAGCA TTCTTAGTAG TGGGTTTCTG 1260
TTCTGTTGGA TGACTTTGAG ATTATCTTT GTTTCCTGTT GGAGTTGTT AAATGTTCTT 1320
TTTAACGGAT GGTGAATGA GCGTCAGCAT CCAGGTTTAT GAATGACAGT AGTCA 1375

```

- (2) INFORMATION FOR SEQUENCE ID NO: 3:  
 (i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 21 nucleotides  
 (B) TYPE: nucleic acid  
 (C) STRANDEDNESS: single  
 (D) TOPOLOGY: linear  
 (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

GACCAGAGTC ATCATGCCTC T

21

- (2) INFORMATION FOR SEQUENCE ID NO: 4:  
 (i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 19 nucleotides  
 (B) TYPE: nucleic acid  
 (C) STRANDEDNESS: single  
 (D) TOPOLOGY: linear  
 (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

TCCTCCACTG ATCTTTACC

19

- (2) INFORMATION FOR SEQUENCE ID NO: 5:  
 (i) SEQUENCE CHARACTERISTICS:  
     (A) LENGTH: 12 amino acid residues  
     (B) TYPE: amino acid  
     (D) TOPOLOGY: linear  
 (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

Lys Ile Ser Gly Gly Pro Arg Ile Ser Tyr Pro Leu  
                             5                            10

- (2) INFORMATION FOR SEQUENCE ID NO: 6:  
 (i) SEQUENCE CHARACTERISTICS:  
     (A) LENGTH: 15 amino acid residues  
     (B) TYPE: amino acid  
     (D) TOPOLOGY: linear  
 (ix) FEATURE:  
     (D) OTHER INFORMATION: Each Xaa may be any amino acid. One, two or all of the N-terminus and C-terminus Xaa moieties may be absent.  
 (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:

Xaa Xaa Xaa Ile Xaa Xaa Xaa Xaa Arg Xaa Xaa Tyr Xaa Xaa Xaa  
                             5                            10                            15

- (2) INFORMATION FOR SEQUENCE ID NO: 7:  
 (i) SEQUENCE CHARACTERISTICS:  
     (A) LENGTH: 9 amino acids  
     (B) TYPE: amino acid  
     (D) TOPOLOGY: linear  
 (ix) FEATURE:  
     (D) OTHER INFORMATION: Each Xaa may be any amino acid.  
 (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 7:

Ile Xaa Gly Xaa Pro Arg Xaa Xaa Tyr  
                             5

- (2) INFORMATION FOR SEQUENCE ID NO: 8:  
 (i) SEQUENCE CHARACTERISTICS:  
     (A) LENGTH: 9 amino acids  
     (B) TYPE: amino acid  
     (D) TOPOLOGY: linear  
 (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 8:

Ile Ser Gly Gly Pro Arg Ile Ser Tyr  
                             5

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(2) INFORMATION FOR SEQUENCE ID NO: 9:

- (i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 10 amino acids  
 (B) TYPE: amino acid  
 (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 9:

Lys Ile Ser Gly Gly Pro Arg Ile Ser Tyr  
                   5                  10

We claim:

1. Isolated peptide consisting of amino acid sequence  
Xaa Ile (Xaa)<sub>4</sub> Arg (Xaa)<sub>2</sub> Tyr Xaa  
wherein the first Xaa is from 0-3 amino acids in length,  
the fourth Xaa is 0-2 amino acids in length, and the first  
and fourth Xaa are no more than 3 amino acids in total,  
each Xaa is any amino acid, and said peptide binds to an  
HLA-Cw16 molecule.
2. The isolated peptide of claim 1 consisting of  
amino acid sequence  
Ile (Xaa)<sub>4</sub> Arg (Xaa)<sub>2</sub> Tyr.
3. The isolated peptide of claim 1, of formula  
Ile (Xaa)<sub>4</sub> Arg (Xaa)<sub>2</sub> Tyr  
wherein the second Xaa in (Xaa)<sub>4</sub> is Gly.
4. The isolated peptide of claim 1, of formula  
Ile (Xaa)<sub>4</sub> Arg (Xaa)<sub>2</sub> Tyr  
wherein the fourth Xaa in (Xaa)<sub>4</sub> is pro.
5. The isolated peptide of claim 1, of formula:  
Ile Xaa Gly Xaa Pro Arg (Xaa)<sub>2</sub> Tyr.
6. The isolated peptide of claim 2, wherein said  
peptide is  
Ile Ser Gly Gly Pro Arg Ile Ser Tyr.
7. The isolated peptide of claim 1, wherein said  
peptide is:  
Lys Ile Ser Gly Gly Pro Arg Ile Ser Tyr Pro Leu.
8. The isolated peptide of claim 1, wherein said  
peptide is  
Lys Ile Ser Gly Gly Pro Arg Ile Ser Tyr.



9. The isolated peptide of claim 1, wherein said HLA-Cw16 molecule is HLA-Cw\*1601.

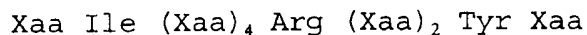
10. Method for provoking proliferation of a cytolytic T cell specific for a complex of a peptide and an HLA-Cw16 molecule, comprising contacting a T cell containing sample with a cell which present complexes of an HLA-Cw16 molecule and the peptide of claim 1 on its surface, under conditions favoring recognition of said complex by a T cell, followed by proliferation.

11. The method of claim 10, wherein said cell is a naturally occurring human cell.

12. The method of claim 11, wherein said cell is a cancer cell.

13. The method of claim 10, wherein said cell is a cell which has been transformed or transfected with a nucleic acid molecule which encodes an HLA-Cw16 molecule.

14. The method of claim 10, wherein said cell is a cell which has been transformed or transfected with a nucleic acid molecule which encodes at least a portion of MAGE-6 tumor rejection antigen precursor, said portion encoding at least a peptide of formula:



wherein the first Xaa is from 0 to 3 amino acids in length, the fourth Xaa is from 0-2 amino acids in length, each Xaa is any amino acid and the first and fourth Xaa are no more than 3 amino acids in total.

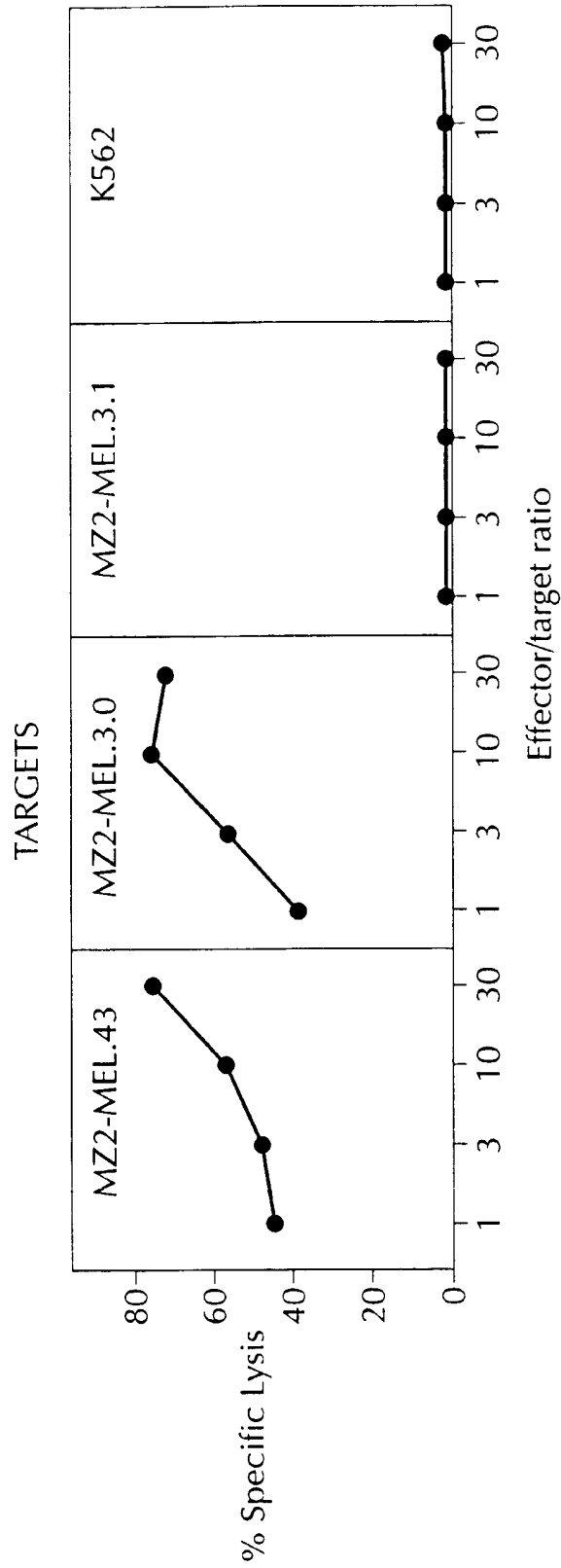
15. Isolated nucleic acid molecule consisting of nucleotides 1033 through 1104 of cDNA for MAGE-6.

16. Expression vector comprising the isolated nucleic acid molecule of claim 15, operably linked to a promoter.

17. Cell line or cell strain, transformed or transfected with the expression vector of claim 16.

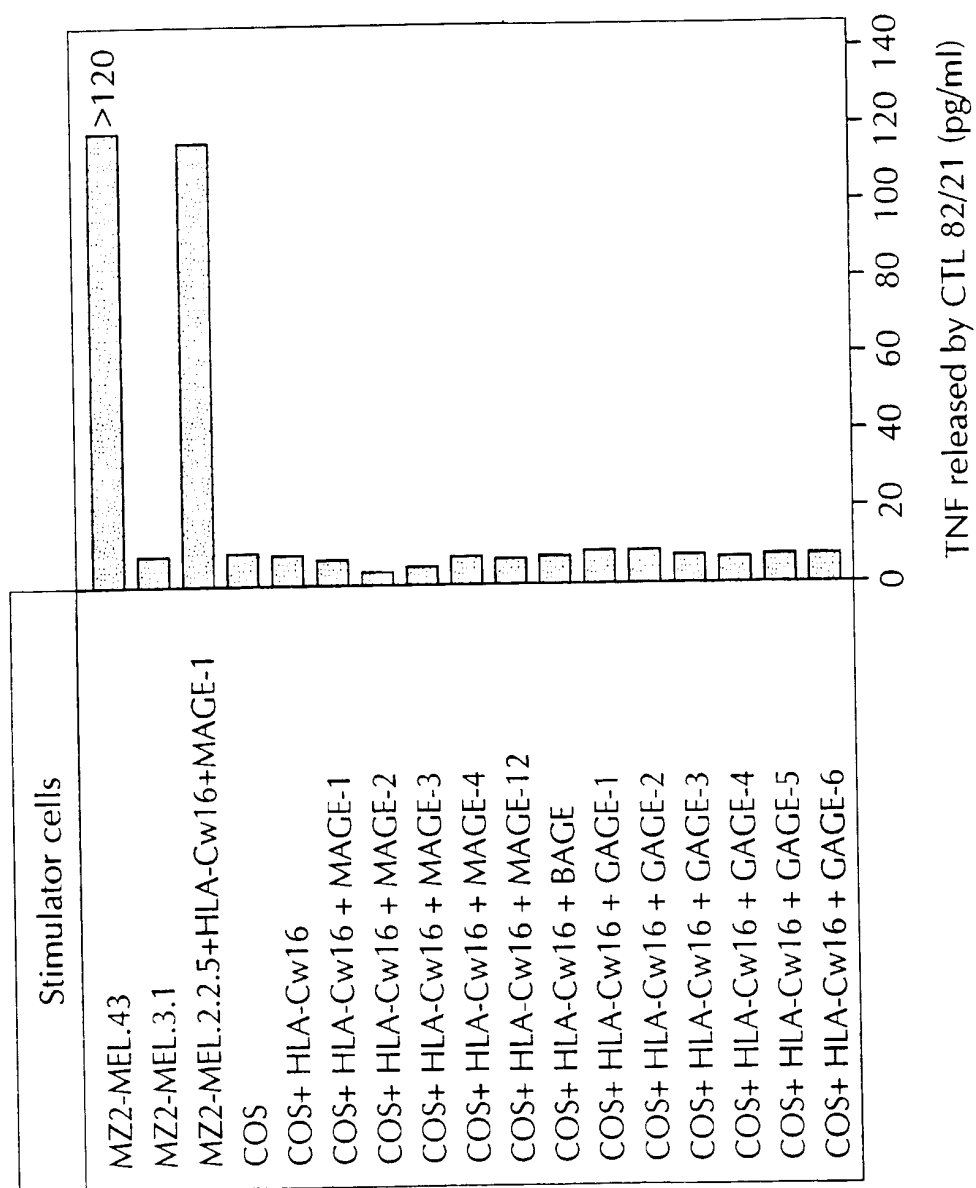
1/3

FIG. 1



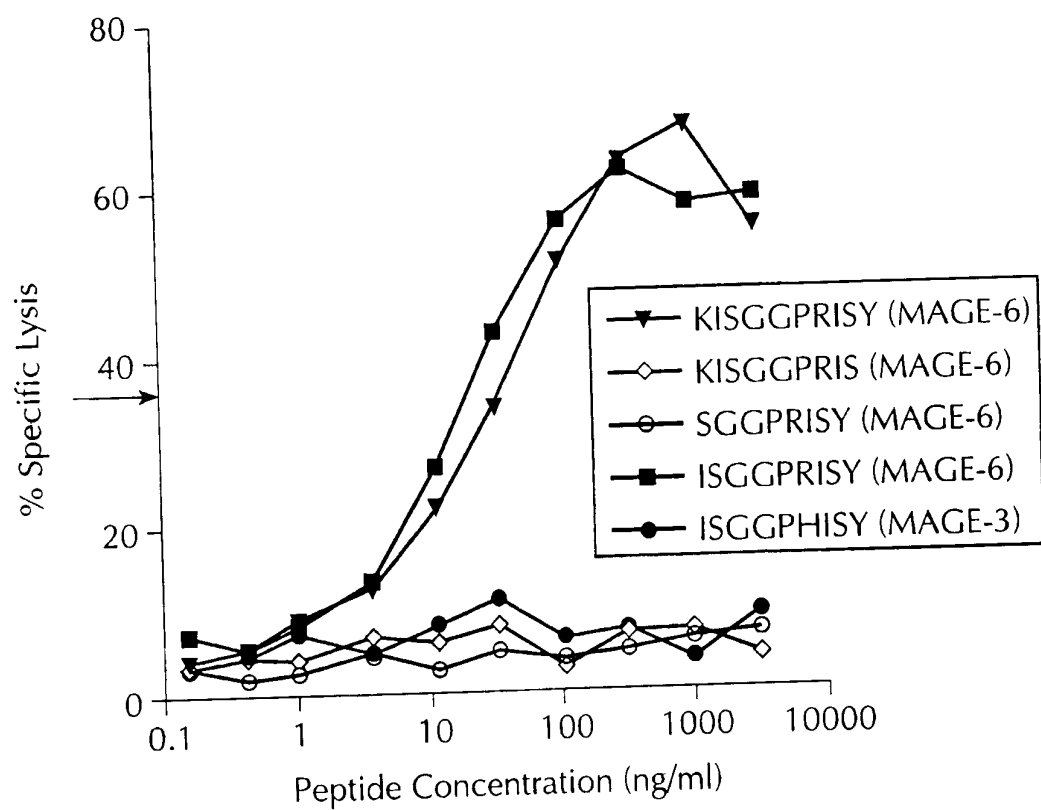
2/3

FIG. 2



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FIG. 3



## INTERNATIONAL SEARCH REPORT

International application No.:

PCT/US97/15069

## A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :A61K 38/08; C12Q 1/68; C12N 15/85,15/86; C07H 21/04; G01N 33/577  
US CL :424/277.1; 435/6, 7.21, 325; 536/23.5

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 424/277.1; 435/6, 7.21, 325; 536/23.5

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched  
NONE

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, MEDLINE, EMBASE, BIOSIS, DERWENT WORLD PATENT

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	VAN DER BRUGGEN et al. Autologous Cytolytic T Lymphocytes Recognize A MAGE-1 Nonapeptide On Melanomas Expressing HLA-CW 1601. Eur. J. Immunol. 1994. Vol. 24. pages 2134-2140, see entire article.	1-17
Y	IMAI et al. Sequence Analysis Of The MAGE Gene Family Encoding Human Tumor-Rejection Antigens. Gene. 1995. Vol. 160. pages 287-290, see entire article.	1-17
Y	DING et al. Cloning And Analysis of MAGE-1-Related Genes. Biochem. and Biophysical Research Commun. 1994. Vol. 202. No. 1. pages 549-555, see entire article.	1-17



Further documents are listed in the continuation of Box C.



See patent family annex.

* Special categories of cited documents:	*T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
*A* document defining the general state of the art which is not considered to be of particular relevance	*X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
*B* earlier document published on or after the international filing date	*Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
*L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	*Z* document member of the same patent family
*O* document referring to an oral disclosure, use, exhibition or other means	
*P* document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search

30 OCTOBER 1997

Date of mailing of the international search report

17 DEC 1997

Name and mailing address of the ISA/US  
Commissioner of Patents and Trademarks  
Box PCT  
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## INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US97/15069

## C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	De PLAEN et al. Structure, Chromosomal Localization, And Expression Of 12 Genes Of The Mage Family. Immunogenetics. 1994. Vol. 40. pages 360-369, see entire article.	1-17
Y	US 5,487,974 (BOON-FALLEUR et al.) 30 January 1996. see entire patent	1-17